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(54) Title: GLUCANS WITH IMMUNOSTIMULANT ACTIVITY (57) Abstract <p>The present invention refers to immunostimulant glucans, to a process for their preparation and to pharmaceutical compositions containing them.</p>		

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GLUCANS WITH IMMUNOSTIMULANT ACTIVITY

The present invention refers to immunostimulant glucans, to a process for their preparation and to pharmaceutical compositions containing them.

Glucan is a polysaccharide occurring in nature in
5 the cell wall of fungine microorganism, particularly of yeasts.

Glucans from different sources, e.g. from different microorganisms, are different one from the other and moreover different extraction processes and
10 treatments to which said microorganisms are subjected, including cultural and maintenance conditions, yield different final products. These differences can be noticed both in the three-dimensional structure of the polysaccharide chain, or in the chemical bonds between
15 glucopyranoside units of said chain, and in the biological activity of the glucans as well as in the presence of substances other than glucan in the crude product with consequently greater or lesser difficulties in the purification.

20 Glucans from Saccharomyces cerevisiae or from Lentinus edodes, both having a branched structure with predominance of β -1,3-glucopyranoside bonds, are known.

Said glucans are particularly studied because of their antitumoral and antibacterial activity (Int. J. Cancer 24, 773-779 (1979); Int. J. Immunopharmacol. Vol. 7 No. 5, 747-751 (1985)). Furthermore, they
25 exhibit an immunomodulator effect both in vivo and in vitro (Rev. Microbiol. Vol. 15, 87-96, 1987) and exert a radioprotective action (Methods and Findings Exptl.

Pharmacol. Vol. 8, No. 3, 151-155 (1986)).

Glucans have been produced also starting from Candida albicans and the immunomodulator effect thereof has been studied (J. Gen. Microbiol. Vol. 134, 1265-74 (1988)).

EP-A-0416343 (16.08.1990) discloses the preparation of parietal glucanic bodies consisting of at least 90% glucan and partly of chitin, by extraction from the strain of Candida albicans ATCC 20955.

The process for the preparation of this product, the biological properties of which are not described and which is in fact described as an intermediate useful for the preparation of final products purified to a degree compatible with the pharmaceutical use, comprises the treatment of the cells in autoclave and subsequent repeated extractions with sodium hydroxide and acetic acid at high temperature.

US patent No. 4992540 (12.02.1991) discloses glucans extracted from Saccharomyces cerevisiae as alimentary additives.

We have now found glucans characterized by particularly high immunostimulant activity and by a remarkable safety thanks to the absence of impurities which are often associated with the similar products until now used.

The glucans of the invention have the following characteristics:

- ratio between $\beta(1-3)$ and $\beta(1-6)$ bonds equal to about 1:1;
- chitin content from 3 to 5% by weight;
- residual protein content lower than 0.3%;

- absence of mannane;
- enhancing activity of the in vitro NK cytotoxic activity.

The glucans of the invention are obtainable by
5 different yeast species.

Although the use of Candida albicans ATCC 20955,
disclosed in EP 0416343, is preferred, the glucans of
the inventions may be obtained from a number of
different strains of Candida, Saccharomyces or other
10 yeast or mycetes species.

The extraction process of the glucans of the
invention from the cells comprises the following steps:

- a) culture of the microorganism in liquid medium,
with low glucose content;
- 15 b) treatment of the cell mass in autoclave at
temperatures higher than 100°C;
- c) repeated extractions with sodium hydroxide and
diluted organic acid;
- d) treatment of the extract with detergent at high
20 temperature.

While steps a)-c) are substantially similar to
that described in EP0416343, the step d) has never been
described and contributes to the peculiar
characteristics of the glucans of the invention.

25 These characteristics particularly comprise high
immunostimulant activity, higher than that of known
glucans, low toxicity and immunogenic activity.

The treatment with detergent at high temperature
is typically carried out using sodium 1-5% dodecyl
30 sulfate, preferably about 2%, in a suitable buffer,
from 1-3 hours at the boiling temperature. The step b)

is preferably carried out at the temperature of about 120°C for 3 hours.

The alternate extractions with NaOH and organic acids such as acetic acid are carried out at
5 temperatures ranging from 80 to 100°C for 24 hours.

Each extraction is followed by washes with water up to neutrality. The alternate extractions with sodium hydroxide and acetic acid are preferably repeated twice, so as to provide an optimal removal of the
10 parietal alkali-soluble glucan and of all the cellular components.

According to a preferred embodiment of the invention, the glucans are prepared starting from Candida albicans strain ATCC 20955. This strain,
15 univocally identified by means of the restriction polymorphism analysis of the cell DNA, has been deposited by the applicants on August 4, 1989 at the American Type Culture Collection according to the Budapest Treaty. As it is known, the safest and most
20 modern method to identify the biotype under exam is the cell DNA restriction polymorphism analysis (DNA restriction fragment length polymorphism; Magee et al. Mol. Cell. Biol. 8, 4721, 1988).

The restriction pattern of the strain provides a
25 genetic fingerprint of the microorganism and turns out to be different from that of all the other members of the Candida genus.

The cultural, biochemical and biological characteristics of the strain are reported hereinbelow:

30 Cultural

characteristics: formation of chlamydospore on corn-

meal agar.

Biochemical

characteristics: it ferments glucose and maltose with production of acid and gas, saccharose with the production of acid only and it does not ferment lactose.

Biological

characteristics: it is pathogenic for rabbit and mouse.

The Candida albicans strain ATCC 20955 is kept on Sabouraud agar Difco in refrigerator at 4°C after 24 h growth at 28°C. For the production, the yeast is grown on a medium having a low glucose content so as to favour the production of the cell-wall, e.g. Winge medium, containing glucose and yeast extract, at 28°C for 18-24 hours, monitoring the culture and checking for the presence of the yeast phase only, so as to obtain an optimal glucose-chitin ratio of approximately 20:1.

The cells grown in the culture broth are collected by centrifugation, washed three times with sterile distilled water and suspended again (1-2% w/v) in pH 5 citrate buffer and then placed in autoclave at 121°C for 3 hours so as to cause the rupture of the cells, the solubilization of the fraction consisting of mannan, proteins, mannoproteins and the release of most cell components.

The mass is collected by centrifugation, resuspended (1%-2% w/v) in 1% sterile NaOH and heated to 100°C for 24 hours. The mass is then washed three

times with sterile distilled water until neutral reaction and then resuspended (1-2% w/v) in sterile 0.5 M acetic acid and treated at 80°C for 24 hours after having being washed three times with sterile distilled water until neutral reaction.

In order to assure an optimal removal of all the protein components which may be dangerous for the therapeutic use, the obtained glucan is further purified by treatment (1-2% w/v suspension) with a 2% sodium dodecylsulfate solution in Tris EDTA mercaptoethanol for 1,5 hours at the boiling temperature.

The product is washed by centrifugation with sterile distilled water until all the detergent is removed. The obtained glucan may also be sterilized in autoclave at 121°C for 30 minutes and finally it is freeze-dried. The obtained product is insoluble in water, methanol, acetone, ethyl ether, diluted acids and alkali, partially soluble in warm 1 M NaOH (0,06%) and soluble in dimethylsulfoxide; it contains 95-97% glucan together with 3-5% of chitin with a protein content lower than 0.3% (usually from 0.1 to 0.3%) and complete absence of mannan: both the IR and ¹³C-NMR (75 MHz at 72°C) spectra show that the polymer is a polysaccharide bound with β (1-3)bonds to form long straight chains from which side chains bound to the main chain through β (1-6) bonds originate. The ratio between β (1-3) and β (1-6) bonds is about 1:1. Also this feature influences the biological activity of the drug since it is reported that the immunological properties of the product are evidently modified by changing

significantly this proportion (Jong SC et al.. EOS-J Immunol. Immunopharmacol. 11(3), 115, 1991). From the spectra, the presence of chitin, which is bound to the structure by covalent bond, is also evident (Kogan G. et al.. Biopolymers 27, 1055, 1988). Thin layer or paper chromatography show the presence of glucose and the absence of mannose, whereas the hexoses titer is 95-97%.

The so obtained glucans are not antigenic and exhibit biological activities which classify them as Biological Response Modifiers. Particularly, studies carried out on mice showed that the administration of the glucans can induce an increase of the anti-infective activity induced by polymorphonucleates leukocytes and activated macrophages both against chronic and acute infection; they also enhance the antitumor activity due to NK cell and activated macrophages; they significantly increase the interleukin production and particularly that of tumor necrosis factor α and interleukin 2; they potentiate the antibody response.

The immunoadjuvant activity in the animal by the parenteral route is very high without remarkable side-effects being noticed and also by the oral route the activity is very interesting, above all as far as the activity on the lung alveolar macrophages is concerned, also perfectly tolerated.

The high purity of the glucans of the invention, mainly in relation to the protein content, imparts to the molecule particularly interesting characteristics from the point of view of tolerability: acute and

chronic toxicity tests did not show any toxic local or systemic effect ($LD_{50} > 1000$ mg/kg i.p. in the mouse and in the rat an $LD_{50} > 2000$ mg/kg p.o. in the mouse and in the rat, no toxic effect after daily
5 administrations repeated for one year with doses up to 400 mg/kg/die or up to 250 mg/kg/die i.p.). Moreover no mutagenic, teratogenic, embryotoxic properties or anyhow influencing fertility have been noticed.

The following Example further illustrate the
10 invention.

EXAMPLE

Candida albicans strains ATCC 20955, maintained on Sabouraud agar slope Difco (glucose 20%, peptone 10%, agar 1.5%, in distilled water, pH 6.5) where grown to a
15 confluent patina in 1-2 days at 28°C, and conserved at ambient temperature or at 4°C. For production, a loopful of C. albicans agar is inoculated in 100 ml of Winge broth (Difco glucose 0.3%, 0.1% Difco yeast extract in distilled water, pH 6.5). The organism was
20 grown at 28°C, under slight stirring (50 rpm) for 18-24 hours until the stationary growth phase was reached (about 2.8×10^8 cells/ml, corresponding to approx. 14 mg of dry weight/ml).

100 ml of broth culture is used to inoculate 1000
25 ml of Winge medium that are incubated as mentioned above.

1000 ml of broth culture previously obtained are used to inoculate 10 l of Winge medium contained in the fermenter. The yeast was grown at 28°C, slightly
30 stirred to 50 rpm, with a stream of air of 1 l/min. and the pH set on 6.5, until the stationary phase of growth

was reached (about $2.8-4.5 \times 10^8$ cells/ml in 24 h). Control were performed during the growth to verify the presence of yeast cells only.

5 The cells grown in the broth culture are harvested by low speed centrifugation (3000 rpm, 30 min), washed three times with distilled sterile water and re-suspended in citrate pH 5 buffer (223 g of citrate sodium/l of distilled water) at a concentration of 2-4% and the suspension is autoclaved for 3 hours at 121°C.

10 The mass is harvested for centrifugation, re-suspended in 1% sterile NaOH at a concentration of about 2-4% and treated for 24 hours in an oil bath at 100°C. The mass is then washed three times via centrifugation with sterile distilled water (neutral
15 reaction) and it is harvested by centrifugation (5000 rpm, 30 min), re-suspended in sterile 0.5 M acetic acid at a concentration of about 2-4% and treated for 24 hours in an oil bath at 80°C.

The mass is washed again three times by
20 centrifugation with sterile distilled water (neutral reaction).

The alternate treatment with sodium hydroxide and acetic acid is repeated twice.

The mass is harvested by centrifugation, re-
25 suspended at a ratio of about 2-4% in a 2% solution of sodium dodecylsulphate in Tris-EDTA-mercaptoethanol buffer (Tris - 0.1 M, EDTA 5 mM, mercaptoethanol 100 mM, pH 6.8) and boiled for 1.5 hours.

The mass is washed three times by centrifugation
30 with sterile distilled water saline. Possible traces of SDS in glucan are extracted by means of solubilization

in dimethylsulfoxide (DMSO) and extracted with water.

Approx. 1 g of glucan is suspended in 25 ml of DMSO and stirred at 77°C until dissolution. The solution obtained is slightly stirred for 15' thereby adding 65 ml of distilled H₂O. Addition of water provokes precipitation of glucan. The mixture obtained is slightly stirred for 5', after which other 65 ml of distilled water are added. The mixture is then centrifuged for 5' at 3500 rpm, and the supernatant discarded.

More water is added to the precipitate, in small quantity and slightly stirred. The whole procedure is repeated until a total proportion of DMSO/H₂O = 1/19 is obtained. The whole process is therefore repeated using twice the volume of DMSO/H₂O mixture.

The product collected for centrifugation after the last washing is transferred on trays and placed in an oven for 24 hours at 60°C.

The process yield is approx. 1.8-2.2 g of glucan per litre of initial culture broth.

The ¹³C-NMR spectrum of the solubilized product has been recorded in DMSO-d₆ with a Bruker AC 300 apparatus at 75 MHz and 70°C. From the integration of the signals at 103 and 86 ppm corresponding to the β(1-3) bonds and those at 60.7 and 70 ppm corresponding to the β(1-6) bonds a ratio among the two kinds of bonds of about 1:1 is calculated, whereas the known glucans, such as those described in US 4992540, EP 0416343 and in Biopolymers 27: 1055, 1988, the ratio is generally quite different, about 65:35 (β1-3 : β1-6) for the glucan of US 4992540 and 35:65 for that of EP 0416343.

The hexose titre, determined according to the method of Dubois et al.. (Anal. Chem. 28, 350, 1956) is 96.4% (96-97% for the glucan described in US 4992540).

5 One single spot with Rf identical to that of reference glucose is showed in ascending paper chromatography (eluent pyridin-ethyl acetate-water 2:5:7) of the glucan subjected to total acid hydrolysis in trifluoroacetic acid 1M at 100°C for 12 hours.

10 The protein content according to Lowry is about 0.16% (0.78% in the glucan described in US 4992540).

15 In the biological assay, carried out according to the method reported in literature (Marconi P. et al.. Infection and Immunity, 50(1): 297-303, 1985) in repeated tests carried out administering 0.1-1 mg of glucan of the invention or obtained according to the method of US 4992540, by i.p. route, 5 days before the in vitro test carried out against the tumor line NK-sensitive YAC-1, using cell suspensions deriving both from the peritoneal exudate and from the spleen, the results obtained in Tables 1 and 2 are obtained, where
20 an immunoadjuvant activity of the glucan of the invention significantly higher than that of the known product is noticed.

TABLE 1: NK Activity (LU 10) of the peritoneal exudate of mice treated with the glucan of the invention (glucan from *C. albicans*) or with that of the US Patent 4992540 (glucan from *S. cerevisiae*)

Animal No.	Dose of glucan from <i>C. albicans</i> 0.001 mg/mouse	Dose of glucan from <i>S. cerevisiae</i> 0.001 mg/mouse	Dose of glucan from <i>C. albicans</i> 0.01 mg/mouse	Dose of glucan from <i>S. cerevisiae</i> 0.01 mg/mouse
1	15	9	79	45
2	11	14	36	48
3	12	11	33	41
4	16	11	40	53
5	4	3	19	30
6	20	14	28	18
7	25	25	99	37
8	9	18	22	26
Mean	14.00	13.13	44.50	37,25
d.s.	6.55	6.49	28.82	11.88
Statistic	Non-significant differences		Non-significant differences	
Test	Wilcoxon test paired data		Wilcoxon test paired data	

TABLE 1 (continued)

Animal No.	Dose of glucan from <i>C. albicans</i> 0.1 mg/mouse	Dose of glucan from <i>S. cerevisiae</i> 0.1 mg/mouse	Dose of glucan from <i>C. albicans</i> 1 mg/mouse	Dose of glucan from <i>S. cerevisiae</i> 1 mg/mouse
1	143	48	212	211
2	377	67	218	62
3	209	86	314	108
4	132	56	123	195
5	130	49	240	154
6	188	59	250	138
7	408	101	461	172
8	84	72	56	80
Mean	208.88	67.25	234.25	140.00
d.s.	119.77	18,56	121.28	53.45
Statistic	Significant differences (p<0.05)		Significant differences (p<0.05)	
Test	Wilcoxon test paired data		Wilcoxon test paired data	

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TABLE 2: NK Activity (LU 10) of spleen cells of mice treated with the glucan of the invention (glucan from *C. albicans*) or with that of the US Patent 4992540 (glucan from *S. cerevisiae*)

Animal No.	Dose of glucan from <i>C. albicans</i> 0.001 mg/mouse	Dose of glucan from <i>S. cerevisiae</i> 0.001 mg/mouse	Dose of glucan from <i>C. albicans</i> 0.01 mg/mouse	Dose of glucan from <i>S. cerevisiae</i> 0.01 mg/mouse
1	576	270	629	274
2	284	220	356	267
3	425	367	718	485
4	223	167	386	327
5	764	1458	776	1817
6	1077	816	1272	864
7	642	542	745	538
8	804	698	728	1195
Mean	599.38	567.25	701.25	720.88
d.s.	285.60	428.00	282.35	547.29
Statistic	Non-significant differences		Non-significant differences	
Test	Wilcoxon test paired data		Wilcoxon test paired data	

TABLE 2 (continued)

Animal No.	Dose of glucan from <i>C. albicans</i> 0.1 mg/mouse	Dose of glucan from <i>S. cerevisiae</i> 0.1 mg/mouse	Dose of glucan from <i>C. albicans</i> 1 mg/mouse	Dose of glucan from <i>S. cerevisiae</i> 1 mg/mouse
1	741	248	409	481
2	381	546	733	409
3	1208	633	1485	802
4	766	543	1412	1073
5	2824	2653	2141	1204
6	1368	902	3620	1070
7	1184	560	1461	820
8	1268	759	1514	1411
Mean	1217.50	855.50	1596.88	908.75
d.s.	730.74	750.41	972.37	347.32
Statistic	Significant differences ($p < 0.05$)			
Test	Wilcoxon test paired data			
	Significant differences ($p < 0.05$)			
	Wilcoxon test paired data			

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In view of the above reported pharmacological properties and of the favourable pharmacological characteristics, mainly as far as tolerability and absence of hypersensitization and anaphylaxis phenomena are concerned, the glucans of the invention may be used for the treatment of tumoral diseases, bacterial or viral infections or of any condition in which a modulation of the immune system is desired. For this purpose, the glucans will be administered in form of pharmaceutical compositions suited to the oral, parenteral, rectal or topical administration. Examples of these formulations comprise tablets, capsules, sachets, syrups, solutions, vials, creams, gels, sprays and the like. The daily dosage will be determined by physicians according to the pathologies to be treated and to the patient's condition (weight, sex, age). It will be usually comprised between 0.1 and 50 mg/kg/die in one or more administrations.

CLAIMS

1. Glucans having the following characteristics:
 - ratio between $\beta(1-3)$ and $\beta(1-6)$ bonds equal to
5 about 1:1;
 - chitin content from 3 to 5% by weight;
 - residual protein content lower than 0.3%;
 - absence of mannan;
 - enhancing activity of the in vitro NK cytotoxic
10 activity.
2. Glucans according to claim 1 obtainable from yeast cells.
3. Glucans according to claim 2 obtainable from Saccharomyces cerevisiae or Candida albicans strains.
- 15 4. Glucans according to claim 3 obtainable from Candida albicans ATCC 20995.
5. A process for the preparation of the glucans of claims 1-4 comprising:
 - a) culture of the microorganism in liquid medium,
20 with low glucose content;
 - b) treatment of the cell mass in autoclave at temperatures higher than 100°C;
 - c) repeated extractions with sodium hydroxide and diluted organic acid;
 - 25 d) treatment of the extract with detergent at high temperature.
6. A process according to claim 5, in which the detergent is 1-5% sodium dodecylsulfate.
7. Use of the glucans of claim 1-5 for the
30 preparation of medicaments having immunostimulating activity.

8. Pharmaceutical compositions containing as the active principle the glucans of claims 1-5 in admixture with a suitable carrier.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP 93/02063

A. CLASSIFICATION OF SUBJECT MATTER

IPC5: C08B 37/00, C12P 19/04, A61K 31/715
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC5: C08B, C12P, A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CA

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO, A1, 9103495 (ALPHA BETA TECHNOLOGY, INC.), 21 March 1991 (21.03.91), see the claims; page 9, line 13 - line 22 --	1-8
X	EP, A2, 0416343 (CONSIGLIO NAZIONALE DELLE RICERCHE ET AL), 13 March 1991 (13.03.91) --	1-6
X	US, A, 4992540 (S. JAMAS ET AL), 12 February 1991 (12.02.91) -- -----	1-6

☐ Further documents are listed in the continuation of Box C.☒ See patent family annex.

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INTERNATIONAL SEARCH REPORT

Information on patent family members

01/10/93

International application No.

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Patent document cited in search report		Publication date	Patent family member(s)		Publication date
WO-A1-	9103495	21/03/91	AU-A-	6441190	08/04/91
			CA-A-	2066172	09/03/91
			EP-A-	0490995	24/06/92
			JP-T-	5503952	24/06/93

EP-A2-	0416343	13/03/91	CA-A-	2023496	05/03/91
			JP-A-	3119995	22/05/91

US-A-	4992540	12/02/91	US-A-	5037972	06/08/91
			US-A-	4810646	07/03/89
			US-A-	5082936	21/01/92